



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Identification of stable reference genes for quantitative PCR in cells derived from chicken lymphoid organs

Citation for published version:

Borowska, D, Rothwell, L, Bailey, RA, Watson, K & Kaiser, P 2016, 'Identification of stable reference genes for quantitative PCR in cells derived from chicken lymphoid organs', *Veterinary Immunology and Immunopathology*, vol. 170, pp. 20-4. <https://doi.org/10.1016/j.vetimm.2016.01.001>

Digital Object Identifier (DOI):

[10.1016/j.vetimm.2016.01.001](https://doi.org/10.1016/j.vetimm.2016.01.001)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Veterinary Immunology and Immunopathology

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



1 Identification of stable reference genes for quantitative PCR in cells
2 derived from chicken lymphoid organs.

3
4 Borowska D.¹, Rothwell L.¹, Bailey R. A.², Watson K.², Kaiser P.¹

5
6
7 ¹The Roslin Institute and R(D)SVS, University of Edinburgh, Easter Bush, Midlothian, EH25 9RG,
8 United Kingdom

9
10 ²Aviagen Ltd, Edinburgh EH28 8SZ, United Kingdom

11
12
13 Corresponding author: Dominika Borowska

14 Phone: (+44) 0131 651 9213

15 Fax: +44 (0) 131 651 9105

16 E-mail: dominika.borowska@roslin.ed.ac.uk
17
18
19
20

Abstract

Quantitative polymerase chain reaction (qPCR) is a powerful technique for quantification of gene expression, especially genes involved in immune responses. Although qPCR is a very efficient and sensitive tool, variations in the enzymatic efficiency, quality of RNA and the presence of inhibitors can lead to errors. Therefore, qPCR needs to be normalised to obtain reliable results and allow comparison. The most common approach is to use reference genes as internal controls in qPCR analyses. In this study, expression of seven genes, including β -actin (ACTB), β -2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -glucuronidase (GUSB), TATA box binding protein (TBP), α -tubulin (TUBAT) and 28S ribosomal RNA (r28S), was determined in cells isolated from chicken lymphoid tissues and stimulated with three different mitogens. The stability of the genes was measured using geNorm, NormFinder and BestKeeper software. The results from both geNorm and NormFinder were that the three most stably expressed genes in this panel were TBP, GAPDH and r28S. BestKeeper did not generate clear answers because of the highly heterogeneous sample set. Based on these data we will include TBP in future qPCR normalisation. The study shows the importance of appropriate reference gene normalisation in other tissues before qPCR analysis.

Keywords: Reference gene; Chicken; Normalisation; Lymphoid tissues; qPCR

Introduction

Transcriptional regulation in response to infections has been studied using different techniques, for example northern blotting, cDNA microarrays, *in situ* hybridisation and quantitative PCR (qPCR) (Matulova *et al.*, 2013; Sandford *et al.*, 2012; Bojesen *et al.*, 2004). The last technique become a very popular tool in host-pathogen interaction studies because of its high sensitivity and potential for high throughput and enhanced specificity. These characteristics are important in immunological research where genes of interest frequently have many splice variants and very low expression levels (Huggett *et al.*, 2005). It is therefore a very useful technique, especially in chicken immunology, where species-specific antibodies are generally not yet commercially available. Although qPCR is the most relevant technique, there are still many problems associated with its use,

mainly inherent variability of RNA, and differences in efficiencies of reverse transcription (RT) and PCR (Bustin, 2002). To make analysis of qPCR reliable, the data need to be normalised using reference genes (also known as internal controls or housekeeping genes). The process of selecting internal control genes needs to be cogent to avoid errors in interpreting the mRNA quantification results (Gantasala *et al.*, 2013). Reference genes usually have well-characterised and permanent functions and, in theory, their expression is stable. Ideal reference genes have consistent expression in varying experimental and environmental conditions. Expression of target genes can be normalised with internal control genes in samples that vary in qualities and quantities of starting RNA. It also compensates for differences in enzymatic efficiencies in individual templates because the reference genes undergo the same preparation steps and are exposed to the same treatments as the gene of interest. An ideal reference gene is yet to be identified (Bär *et al.*, 2009). Many authors suggest that a definite or universal internal control gene for every condition in different tissues and cells does not exist (Maltseva *et al.*, 2013; Coulson *et al.*, 2008; Vandesompele *et al.*, 2002). There are increasing numbers of studies on widely used reference genes that prove many of them are not resistant to changes in the experimental environment (Yang *et al.*, 2013; Yin *et al.*, 2011; Sugden *et al.*, 2010; Yue *et al.*, 2010; He *et al.*, 2008; Bas *et al.*, 2004; Dheda *et al.*, 2004; Lupberger *et al.*, 2002; Schmittgen and Zakrajsek 2000). It has therefore been suggested that determination of appropriate reference genes should be performed for experiments involving a specific cell type or tissue with different experimental settings before qPCR (Riemer *et al.*, 2012).

The use of reference genes as internal controls in qPCR normalisation studies is now a standard procedure. Researchers have used many methods to identify reference genes. The most popular strategies are the use of software and algorithms such as GeNorm (Vandesompele *et al.*, 2002), BestKeeper (Pfaffl *et al.*, 2004) and NormFinder (Andersen *et al.*, 2004). Many studies use more than one of these programs as they differ in their underlying assumptions (Chang *et al.*, 2012; Ledderose *et al.*, 2011; Perez *et al.*, 2008).

In this study, the stability of seven reference genes was measured with the aim of creating a set of genes that could be used as internal controls in mRNA expression studies in chicken lymphoid organs, and to confirm ribosomal 28S (r28S), which we have used as a reference gene in these studies

for fifteen years, was appropriate. A group of standard reference genes (ACTB, B2M, GAPDH, GUSB, TBP, TUBAT, r28S) was chosen for evaluation of their mRNA expression in cells isolated from the spleen, bursa and thymus and stimulated with different mitogens (see Materials and Methods). The three softwares described above were used to calculate gene stability in an effort to select the least variable genes as appropriate controls in future expression studies.

Materials and methods

Tissue-cell collection and stimulation

J-line layer chickens were bred and hatched at The Roslin Institute. Birds were reared in floor pens and water and feed was provided *ad libitum*. Bursa of Fabricius, spleen and thymus were collected from each bird and single-cell suspensions prepared by gently squeezing the tissues through a 40 µm nylon strainer. Leukocytes were isolated with density gradient centrifugation for 20 min at 300 x g using Histopaque 1.077 (Sigma-Aldrich, Poole, UK). Isolated cell numbers were adjusted to 5 x 10⁶ cells/ml with pre-warmed RPMI media containing 10% CS. Cells were cultured in 25 mm² flasks for 4 h with the addition of 500 ng/ml phorbol myristate acetate (PMA) and 1 µg/ml ionomycin for bursal cells, 1 µg/ml Concanavalin A (ConA) for splenocytes and 25 µg/ml phytohaemagglutinin (PHA) for thymocytes.

RNA extraction and cDNA synthesis

Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. The quality and quantity of the extracted total RNA was evaluated by spectrophotometry using NanoDrop™ 1000. First-strand cDNA synthesis was performed using a SuperScript III reverse transcription kit (Paisley, UK) containing random primers (Sigma-Aldrich) and according to the manufacturer's instructions. The cDNA was stored at -20°C until further use.

Gene selection and quantitative PCR

Seven genes commonly used as reference genes in quantitative PCR (qPCR) gene expression experiments were selected: beta-actin (ACTB, structural framework inside cells) (Gunning *et al.*, 1983); beta-2-microglobulin (B2M, part of the major histocompatibility complex) (Güssow *et al.*, 1987); glyceraldehyde-3-phosphate dehydrogenase (GAPDH, related to carbohydrate metabolism) (Sirover, 1997); beta-glucuronidase (GUSB, involved in the breakdown of glycosaminoglycans) (Shipley *et al.*, 1993); TATA box binding protein (TBP, indicates transcription start sites) (White *et al.*, 1992); alpha-tubulin (TUBAT, forms and organizes microtubules) (Ludueña, 1997); 28S ribosomal RNA (28S rRNA, structural RNA for the large component of cytoplasmic ribosomes) (Wool, 1979). All qPCR primers were designed using Primer Express Software 3.0 (Life Technologies) and synthesised by Sigma Aldrich. Primer sequences and amplicon lengths are shown in Table 1.

Reaction mixes were prepared using the following components for each of the samples: 5 µl ABI TaqMan Gene Expression Master Mix (Applied Biosystems, Paisley, UK, 0.5 µl 20X EvaGreen (Biotum, VWR-Bie & Berntsen), 2.5 µl 20 µM specific primer (forward and reverse) and 10 µl of water. Each reaction contained 2 µl of cDNA diluted 1:3 in low EDTA TE buffer. Quantitative PCR was performed with an Applied Biosystems 7500 Fast Real-Time PCR System with the following cycle profile: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles with denaturing for 15 s at 95°C, and annealing/elongation for 1 min at 60°C. Melting curves were generated to confirm a single-PCR product for each reaction (from 60°C to 95°C, increasing 1°C every 3 s). All reactions were performed in duplicate and in each run internal standard curves (serial dilutions of a pooled cDNA sample for each tissue-type, mock and antigen-stimulated) were used to assign relative concentrations to the samples.

Statistical analyses

To select suitable internal controls, the stability of each gene was statistically analysed with three software packages: GeNorm (Vandesompele *et al.*, 2002); NormFinder (Andersen *et al.*, 2004) and BestKeeper (Pfaffl *et al.*, 2004). All three packages were used according to the supplied instructions. The BestKeeper input format requires untransformed quantification cycle (Cq) values. The GeNorm

and NormFinder input formats require gene expression data to be transformed to relative quantities (Q) where Cq values for each particular gene are normalised to the sample with the highest expression level (the lowest Cq) by a delta Cq formula (Equation 1).

$$\Delta Cq = \min Cq (\text{reference sample}) - Cq \text{ sample} \quad (1)$$

Relative quantities (Equation 2) are calculated based on PCR amplification efficiency (E) (Equation 3) that is derived based on the slope of the standard curve, by graphing the log of the DNA concentration used versus Cq value for the sample (Supplementary file).

$$Q = E^{\Delta Cq} \quad (2)$$

$$E = 10^{(-1/\text{slope})} \quad (3)$$

The three software programs generate measures of reference gene stability. geNorm, using Q values, calculates the M value of a given gene based on the arithmetic mean of all pair-wise variations between a particular gene and all other genes examined (Equation 4).

$$M_j = \frac{\sum_{k=1}^n V_{jk}}{n-1} \quad (4)$$

where:

M_j – gene stability measure,

V_{jk} – pairwise variation of gene j relative to gene k,

n – total number of examined genes

geNorm calculates the optimal number of reference genes required for the analysis. Based on the geometric mean of the expression levels, normalisation factors are calculated by stepwise inclusion of an additional reference gene. If the subsequent gene causes a decrease in variation ($V_{n/(n+1)}$), it should be included in the analysis as it has a significant effect. Vandesompele *et al.* (2002) suggest the M value not to be greater than 1.5, where lower values indicate an increase in gene stability and decrease in gene expression variability. The pair-wise comparison approach selects genes based on their degree of similarity. Therefore, candidates with lower M values do not necessarily become top ranked. The

pair-wise stability measure indicates if the addition of extra genes to the normalisation process is beneficial and changes the accuracy of the analysis. NormFinder also relies on Q values to estimate the stability of each gene. Quantities are first log-transformed and then used in an ANOVA model-based approach to calculate expression variation where intra- and inter-group variations are estimated. The two sources of variation represent systematic error that will occur when the given gene will be used (Andersen *et al.*, 2004). BestKeeper analyses the expression stability using descriptive statistics: geometric mean (GM), arithmetic mean (AM), minimal (Min) and maximal (Max) value, standard deviation (SD) and coefficient of variance (CV). CV and SD values are used to determine the stability of the reference gene expression, where the most stably expressed genes have the lowest CV and the SD value is below one. Internal controls with SD higher than one can be regarded as unreliable. The genes that are considered to be stably expressed are used to calculate a BestKeeper Index (BKI) as the geometric mean. BestKeeper also analyses inter-housekeeping gene (HKG) relations using the Pearson correlation coefficient (r) and the probability (p) value.

Results and Discussion

Quantitative PCR is now a standard technique to study RNA expression levels. To precisely determine amplification of transcript fragments, normalisation strategies are necessary (Bustin, 2000). There are several guidelines that can be followed to minimise inaccuracies in gene expression studies. For example, uniform sample size, RNA extraction methods, reduction of gDNA contamination and internal controls. These methods are not mutually exclusive and can all be included in the protocol (Huggett *et al.*, 2005). Using a reference gene as an internal control for amplification of the mRNA is the most commonly used and suitable technique (Radonić *et al.*, 2004). In this study, expression levels of seven reference genes (Table 1) were measured in cells isolated from chicken lymphoid organs (bursa, spleen and thymus) and then stimulated with different mitogens. To identify the most suitable genes for normalisation of qPCR, the geNorm, NormFinder and BestKeeper algorithms were used. All three software programs recognise control genes by determining their expression stability.

NormFinder, using calculated relative quantities, identified TBP as the most stably expressed reference gene with a stability value of 0.070 followed by GAPDH (0.151) and 28S rRNA (0.155) (Figure 1). For the best combination of two reference genes, the program suggested GAPDH and TBP with a stability value of 0.083. Ribosomal RNA 28S performed equally well as GAPDH, whereas B2M and TUBAT were the worst scoring genes in the panel, with stability values of 0.22 and 0.30 respectively.

Relative quantities as input data were also used in the geNorm algorithm (Figure 2A). Analogous to NormFinder, geNorm identified TBP and GAPDH as the two most stable genes with an average gene stability M score of 0.72, which is characteristic for heterogeneous samples, where the acceptable M value should be lower than 1 (Hellemans *et al.*, 2007). The M value for r28S was second lowest after the TBP M value, yet the program chose GAPDH as one of two ideal internal controls in the tissue panel tested. The frequently used housekeeping gene ACTB performed poorly and, according to the geNorm algorithm, should not be used as a reference gene in the samples tested. The pair-wise variation $V_{n/(n+1)}$ for seven reference genes is shown in Figure 2B. The results suggested that three reference genes were sufficient, but the inclusion of a fourth gene did not cause an increase in the variation. Although the pair-wise variation cut-off value (0.15) has not been achieved, using at least three of the most stable reference genes is in agreement with the recommendation from the geNorm software developers.

Opposite to the previously described algorithms, BestKeeper uses a raw qPCR C_q as input data to calculate descriptive statistics. Standard deviation values for all reference genes tested in this experiment were higher than one. Therefore, all genes were disregarded from further analysis. In contrast to geNorm or NormFinder, BestKeeper does not allow ranking of the reference genes using the stability value and it does not suggest an optimal number of reference genes. The studied sample set was very heterogeneous which theoretically invalidated the use of the Pearson correlation coefficient. However, Pfaffl *et al.* (2004) mentioned very high correlation between lower C_q values (UBQ, GAPDH, ACTB) and higher (18S) C_q values, which was the reason not to exclude 18S from their index. BestKeeper and geNorm are based on the same principle. However, the two algorithms do

not always display overlapping reference genes (Cinar *et al.*, 2012). BestKeeper is robust against sampling errors but it requires in-depth knowledge of the co-regulation of the candidate genes (Tong *et al.*, 2009). Considering the BestKeeper statistical methods, modification of candidate gene expression levels by any other gene in the panel could bias the results and any interpretation would be false. Similar problems may be encountered when using the geNorm algorithm, as its pair-wise comparison does not correct for co-regulation. NormFinder calculations, on the other hand, are not influenced by co-regulation (Andersen *et al.*, 2004). Thus, inclusion of genes that represent a cross-section of independent cellular functions should correct for putative co-regulation effects in the same experimental settings (Riedel *et al.*, 2014). NormFinder and geNorm results in this study agree that TBP is the most stably expressed gene among all seven candidates tested. Both softwares indicate GAPDH as the best gene when combined with TBP.

There are few published studies on reference gene normalisation in chicken cells or tissues and all of the existing results differ in their ranking of the genes. Most of the studies have focused on chicken embryo fibroblasts (CEFs) as a virus infection model. The results of Yin *et al.* (2011) indicated ACTB as the most stably expressed gene in CEFs infected with NDV and GAPDH along with 18S as the least stable genes, based on their transcriptional profiles only. Yue *et al.* (2010) used CEFs infected with AIV and in cell response studies YWHAZ was the most stable gene, whereas in virus replication studies ACTB and RPL4 were the most reliable controls according to geNorm. The same software was used to determine the best reference gene in CEFs infected with ALV-J. geNorm ranked RPL30 and SDHA as the best candidates and ACTB and GAPDH as the least stable genes (Yang *et al.*, 2013). de Boever *et al.* (2008) identified GAPDH and UBC together as the best pair of internal controls in cells of chickens stimulated with LPS. In duck and chicken primary lung cells, infected with LPAIV and HPAIV, GAPDH was ranked as the second best reference gene after 18S (Kuchipudi *et al.*, 2012).

In this study, GAPDH and r28S were ranked as the second-most stable reference genes in chicken lymphoid tissues. There have been many reports of GAPDH expression being unstable in other experiments (Barber *et al.*, 2005; Lin *et al.*, 2012; Sudgen *et al.*, 2010). The use of ribosomal

RNA as a normaliser can be controversial, based on its technical limitations and can lead to its exclusion from analyses (Lu *et al.*, 2013). The ubiquitous abundance of ribosomal RNA and lower rate of degradation, compared to mRNA, may influence the results of qPCR (Vandesompele *et al.*, 2002). This is very important for studies on genes characterised with general low abundance where smaller changes in relative expression cannot be detected. The cDNA require dilutions prior to qPCR analysis using a ribosomal reference gene, which may introduce dilution errors. Nevertheless, ribosomal RNAs, including r28S and r18S, has been shown to be stably expressed reference genes (Wang *et al.*, 2011; Røge *et al.*, 2007). Li *et al.* (2005) reported that r28S was among few genes with stable expression in CEFs infected with IBDV but in the same experiment B2M and TBP were the least stable. In *in vitro* stimulation of human blood cells, TBP was a good reference gene in studies on T lymphocytes, neutrophils and total blood leukocytes (Ledderose *et al.*, 2011).

Our own laboratory has used r28S as a reference gene for nearly two decades, as published in more than 50 papers (e.g. Rothwell *et al.*, 2012; Wu *et al.*, 2009; Kogut *et al.*, 2003). This decision was based on early studies in the laboratory, which were never published, comparing expression of r28S, GAPDH, β -actin and ovotransferrin in splenocytes and thymocytes stimulated with a variety of mitogens for various times (Kaspers, Rothwell, Kaiser, unpublished). Ribosomal 28S was by far the most stably expressed of the four genes, and has thus been the laboratory standard housekeeping gene since, until it was decided to revisit the subject with modern analyses.

The current study is the first published report of reference gene normalisation in stimulated chicken lymphoid organ-derived cells. These results demonstrate the need to carefully select reference genes for immune genes expression studies. Although this study showed that TBP, GAPDH and r28S are suitable gene expression normalisers for chicken lymphoid cells, we strongly recommend testing internal control genes before gene expression studies in other chicken tissues or cells.

Acknowledgments

The authors would like to acknowledge Kate Sutton and Louise Welch for providing the RNA samples used in this study. Special thanks to Pip Beard and Ismar Haga for access to the geNorm.

258 Dominika Borowska's PhD studies were funded by Aviagen, the Biotechnology and Biological
259 Science Research Council and through BBSRC Institute Strategic Programmes Grants to The Roslin
260 Institute.

261

References

- Andersen C.L., Jensen J.L., Ørntoft T.F., 2004. Normalisation of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalisation, applied to bladder and colon cancer data sets. *Cancer Research* 64; 5245-5250.
- Bär M., Bär D., Lehman B., 2009. Selection and validation of candidate housekeeping genes for studies of human keratinocytes – review and recommendations. *The Journal of Investigative Dermatology* 129; 535-537.
- Barber R.D., Harmer D.W., Coleman R.A., Clark B.J., 2005. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiological Genomics* 21; 389-395.
- Bas A., Forsberg G., Hammarström S., Hammarström M-L., 2004. Utility of the housekeeping genes 18S rRNA, beta-actin and glyceraldehyde-3-phosphate-dehydrogenase for normalisation in real-time quantitative reverse transcriptase- polymerase chain reaction analysis of gene expression in human T lymphocytes. *Scandinavian Journal of Immunology* 59; 566-573.
- Bojesen A.M., Nielsen O.L., Christensen J.P., Bisgaard M., 2004. *In vivo* studies of *Gallibacterium anatis* infection in chickens. *Avian Pathology* 33; 145-152.
- Bustin S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* 25; 169-193.
- Bustin S.A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology* 29; 23-29.
- Chang E., Shi S., Liu J., Cheng T., Xue L., ang X., Yang W., Lan Q., Jiang Z., 2012. Selection of reference genes for quantitative gene expression studies in *Platycladus orientalis* (cupressaceae) using real-time PCR. *PLoS ONE* 7; e33278.

285 Cinar M.U., Islam M.A., Uddin M.J., Tholen E., Tesfaye D., Looft C., Schellander K., 2012.
 286 Evaluation of suitable reference genes for gene expression studies in porcine alveolar macrophages in
 287 response to LPS and LTA. *BMC Research Notes* 5: 107.

288 Coulson D.T.R., Brockbank S., Quinn J.G., Murphy S., Ravid R., Irvine G.B., Johnston J., 2008.
 289 Identification of valid reference genes for the normalisation of RT qPCR gene expression data in
 290 human brain tissue. *BMC Molecular Biology* 9; 46.

291 de Boever S., Vangestel C., de Backer P., Croubels S., Sys S.U., 2008. Identification and validation of
 292 housekeeping genes as internal control for gene expression in an intravenous LPS inflammation
 293 model in chickens. *Veterinary Immunology and Immunopathology* 122; 312-317.

294 Dheda K., Huggett J.F., Bustin S.A., Johnson M.A., Rook G., Zumla A., 2004. Validation of
 295 housekeeping genes for normalisation RNA expression in real-time PCR. *BioTechniques* 37; 112-114.

296 Gantasala N.P., Papolu P.K., Thakur P.K., KAmaraaju D., Sreevathsa R., Rao U., 2013. Selection and
 297 validation of reference genes for quantitative gene expression studies by real-time PCR in eggplant
 298 (*Solanum melongena* L). *BMC Research Notes* 6; 312.

299 Gunning P., Ponte P., Okayama H., Engel J, Blau H., 1983. Isolation and characterisation of full-
 300 length cDNA clones for human α -, β -, and γ -actin mRNAs: Skeletal but not cytoplasmic actins have
 301 an amino-terminal cysteine that is subsequently removed. *Molecular and Cellular Biology* 3; 787-795.

302 Güssow D., Rein R., Ginjaar I., Hochstenbach F., Seemann G., Kottman A., Ploegh H.L., 1987. The
 303 human β_2 -microglobulin gene. Primary structure and definition of the transcriptional unit. *The Journal*
 304 *of Immunology* 139; 3132-3138.

305 He J-Q., Sandford A.J., Wang I-M., Stepaniants S., Knight D.A., Kicic A., Stick S.M., Paré P.D.,
 306 2008. Selection of housekeeping genes for real-time PCR in atopic human bronchial epithelial cells.
 307 *The European Respiratory Journal* 32; 755-762.

308 Hellemans J., Mortier G., de Paepe A., Speleman F., Vandesompele J., 2007. qBase relative
 309 quantification framework and software management and automated analysis of real-time quantitative
 310 PCR data. *Genome Biology* 8: R19.

311 Huggett J., Dheda K., Bustin S., Zumla A., 2005. Real-time RT-PCR normalisation; strategies and
 312 considerations. *Genes and Immunity* 6; 279-284.

313 Kogut M.H., Rothwell L., Kaiser P., 2003. Differential regulation of cytokine gene expression by
 314 avian heterophils during receptor-mediated phagocytosis of opsonized and nonopsonized *Salmonella*
 315 Enteritidis. *Journal of Interferon and Cytokine Research* 23; 319-327.

316 Kuchipudi S.V., Tellabati M., Nelli R.K., White G.A., Baquero Perez B., Sebastian S., Slomka M.J.,
 317 Brookes S.M., Brown I.H., Dunham S.P., Chang K-C., 2012. 18S rRNA is a reliable normalisation
 318 gene for real time PCR based on influenza virus infected cells. *Virology Journal* 9; 230.

319 Ledderose C., Heyn J., Limbeck E., Kreth S., 2011. Selection of reliable reference genes for
 320 quantitative real-time PCR in human T cells and neutrophils. *BMC Research Notes* 4; 427.

321 Li Y.P., Bang D.D., Handberg K.J., Jorgensen P.H., Zhang M.F., 2005. Evaluation of the suitability
 322 of six host genes as internal control in real-time RT-PCR assays in chicken embryo cell cultures
 323 infected with infectious bursal disease virus. *Veterinary Microbiology* 110; 155-165.

324 Lin J., Redies C., 2012. Histological evidence: housekeeping genes beta-actin and GAPDH are of
 325 limited value for normalisation of gene expression. *Development Genes and Evolution* 222; 369-376.

326 Lu Y., Yuan M., Gao X., Kang T., Zhan S., Wan H., Li J., 2013. Identification and validation of
 327 reference genes for gene expression analysis using quantitative PCR in *Spodoptera litura*
 328 (Lepidoptera: Noctuidae). *PloS ONE* 8: e68059.

329 Ludueña R.F., 1997. Multiple forms of tubulin: Different gene products and covalent modifications.
 330 *International Review of Cytology* 178; 207-275.

331

332 Lupberger J., Kreuser K.A., Baskaynak G., Peters U.R., le Coutre P., Schmidt C.A., 2002.
 333 Quantitative analysis of beta-actin, beta-2-microglobulin and porphobilinogen deaminase mRNA and
 334 their comparison as control transcripts for RT-PCR. *Molecular and Cellular Probes* 16; 25-30.

335 Maltseva D.V., Khaustova N.A., Fedotov N.N., Matveeva E.O., Lebedev A.E., Shkurnikov M.U.,
 336 Galatenko V.V., Schumacher U., Tonevitsky A.G., 2013. High-throughput identification of reference
 337 genes for research and clinical RT-qPCR analysis of breast cancer samples. *Journal of Clinical*
 338 *Bioinformatics* 3; 13.

339 Matulova M., Vermozova K., Sisak F., Havlickova H., Babak V., Stejskal K., Zdrahal Z., Rychlik I.,
 340 2013. Chicken innate immune response to oral infection with *Salmonella enterica* serovar Enteritidis.
 341 *Veterinary Research* 44; 37.

342 Perez R., Tupac-Yupanqui I., Dunner S., 2008. Evaluation of suitable reference genes for gene
 343 expression studies in bovine muscular tissue. *BMC Molecular Biology* 9; 79.

344 Pfaffl M.W., Tichopad A., Prgomet C., Neuvians T.P., 2004. Determination of stable housekeeping
 345 genes, differentially regulated target genes and sample integrity: BestKeeper – Excel-based tool using
 346 pair-wise correlations. *Biotechnology Letters* 26; 509-515.

347 Radonić A., Thulke S., Mackay I.M., Landt O., Siegert W., Nitsche A., 2004. Guideline to reference
 348 gene selection for quantitative real-time PCR. *Biochemical and Biophysical Research*
 349 *Communications* 313; 856-862.

350 Riedel G., Rudrich U., Fekete-Drimusz N., Manns M.P., Vondran F.W.R., Bock M., 2014. An
 351 extended Δ CT-Method facilitating normalisation with multiple reference genes suited for quantitative
 352 RT-PCR analyses of human hepatocyte-like cells. *PloS ONE* 9: e93031.

353 Riemer A.B., Keskin D.B., Reinherz E.L., 2012. Identification and validation of reference genes for
 354 expression studies in human keratinocyte cell lines treated with and without interferon- γ – a method
 355 for qRT-PCR reference gene determination. *Experimental Dermatology* 21; 625-629.

356 Røge R., Thorsen J., Tørring C., Øzbay A., Møller B.K., Carstens J., 2007. Commonly used reference
 357 genes are actively regulated in *in vitro* stimulated lymphocytes. *Scandinavian Journal of Immunology*
 358 65; 202-209.

359 Rothwell L., Hu T., Wu Z., Kaiser P., 2012. Chicken interleukin-21 is costimulatory for T cells and
 360 blocks maturation of dendritic cells. *Developmental and Comparative Immunology* 36; 475-482.

361 Sandford E., Orr M., Shelby M., Li X., Zhou H., Johnson T.J., Kariyawasam S., Liu P., Nolan L.K.,
 362 Lamont S.J., 2012. Leukocyte transcriptome from chickens infected with avian pathogenic
 363 *Escherichia coli* identifies pathways associated with resistance. *Results in Immunology* 2; 44-53.

364 Schmittgen T.D., Zakrajsek B.A., 2000. Effect of experimental treatment on housekeeping gene
 365 expression: validation by real-time, quantitative RT-PCR. *Journal of Biochemical and Biophysical*
 366 *Methods* 46; 69-81.

367 Shipley J.M., Grubb J.H., Sly W.S., 1993. The role of glycosylation and phosphorylation in the
 368 expression of active human β -glucuronidase. *The Journal of Biological Chemistry* 268; 12193-12196.

369 Sirover M.A., 1997. Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in
 370 normal cell function and in cell pathology. *Journal of Cellular Biochemistry* 66; 133–140.

371 Smith R.D., Ogden C.W., Penny M.A., 2001. Exclusive amplification of cDNA template (EXACT)
 372 RT-PCR to avoid amplifying contaminating genomic pseudogenes. *BioTechniques* 31; 776-782.

373 Sugden K., Pariante C.M., McGuffin P., Aitchison K.J., D'Souza U.M., 2010. Housekeeping gene
 374 expression is affected by antidepressant treatment in a mouse fibroblast cell line. *Journal of*
 375 *Psychopharmacology* 24; 1253-1259.

376 Tong Z., Gao Z., Wang F., Zhou J., Zhang Z., 2009. Selection of reliable reference genes for gene
 377 expression studies in peach using real-time PCR. *BMC Molecular Biology* 10:71.

378 Vandesompele J., de Preter K., Pattyn F., Poppe B., van Roy N., Paepe A., Speleman F., 2002.
 379 Accurate normalisation of real-time quantitative RT-PCR data by geometric averaging of multiple
 380 internal control genes. *Genome Biology* 3: research 0034.1-0034.11.

381 Yang F., Lei X., Rodriguez-Palacios A., Tang C., Yue H., 2013. Selection of reference genes for
 382 quantitative real-time PCR analysis in chicken embryo fibroblasts infected with avian leukosis virus
 383 subgroup J. *BMC Research Notes* 6; 402.

384 Yin R., Liu X., Liu C., Ding Z., Zhang X., Tian F., Liu W., Yu J., Hrabe de Angelis M., Stoeger T.
 385 2011. Systematic selection of housekeeping genes for gene expression normalisation in chicken
 386 embryo fibroblasts infected with Newcastle disease virus. *Biochemical and Biophysical Research
 387 Communications* 413; 537-540.

388 Yue H., Lei X-W., Yang F-L., Li M-Y., Tang C., 2010. Reference gene selection for normalisation of
 389 PCR analysis in chicken embryo fibroblast infected with H5N AIV. *Virologica Sinica* 25; 425-431.

390 Wang E-H., Truong L.D., Mendoza L., Jung E.S., Choi Y-J., 2011. 28S-ribosomal RNA is superior to
 391 glyceraldehyde-3-phosphate dehydrogenase as a RNA reference gene in p53-deficient mice with
 392 unilateral ureteral obstruction. *Experimental and Molecular Pathology* 91; 368-372.

393 White R.J., Jackson S.P., Rigby P.W.J., 1992. A role for the TATA-box-binding protein component of
 394 the transcription factor. *PNAS* 89; 1949-1953.

395 Wool I.G., 1979. The structure and function of eukaryotic ribosomes. *Annual Reviews of Biochemistry*
 396 48; 719-754.

397 Wu Z., Rothwell L., Hu T., Kaiser P., 2009. Chicken CD14, unlike mammalian CD14, is trans-
 398 membrane rather than GPI-anchored. *Developmental and Comparative Immunology* 33; 97-104

399
 400

Figure 1. The NormFinder analysis of reference genes showing stability values. Lower stability value indicates gene that is more stable.

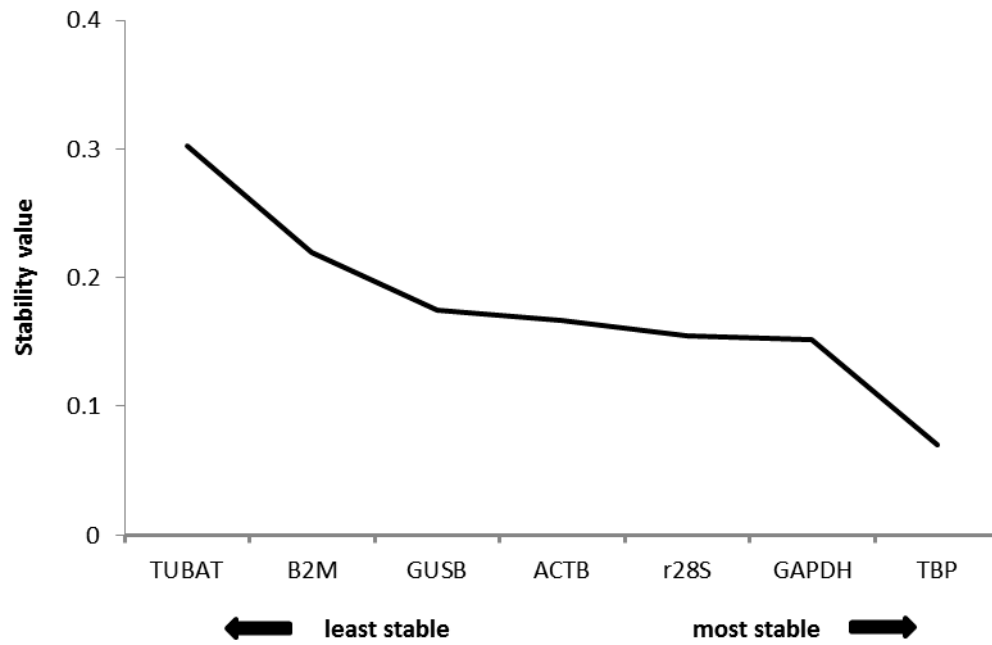
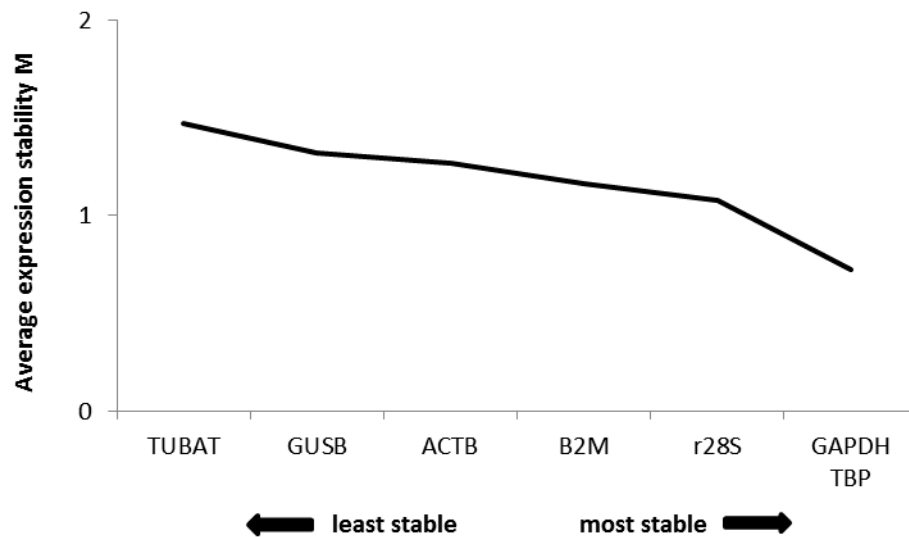


Figure 2. geNorm analysis. A) Average expression stability M of all seven reference genes. The most stably expressed genes have lower M values. B) Optimal number of reference genes required for reliable normalisation calculated by pair-wise variation analysis between normalisation factors NFn and $NFn+1$. According to geNorm, addition of a fourth gene has significant effect.

A



B

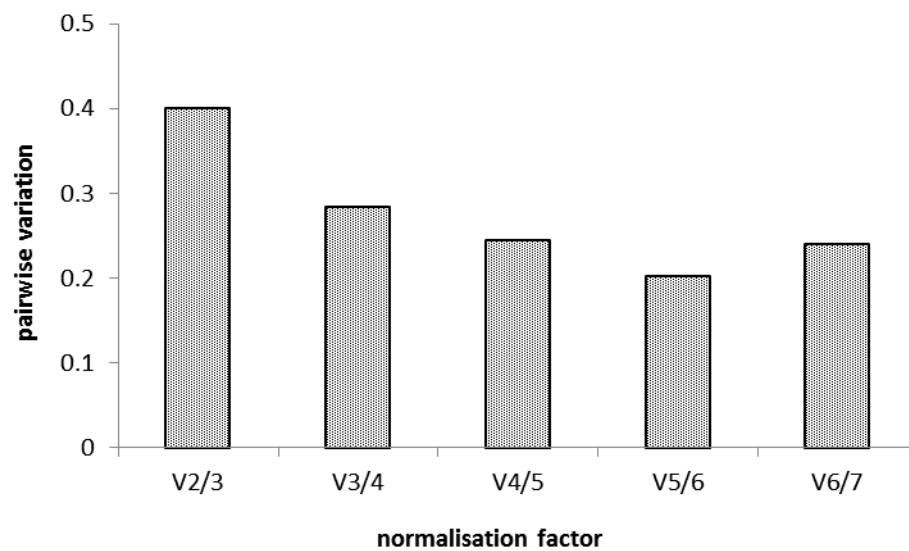
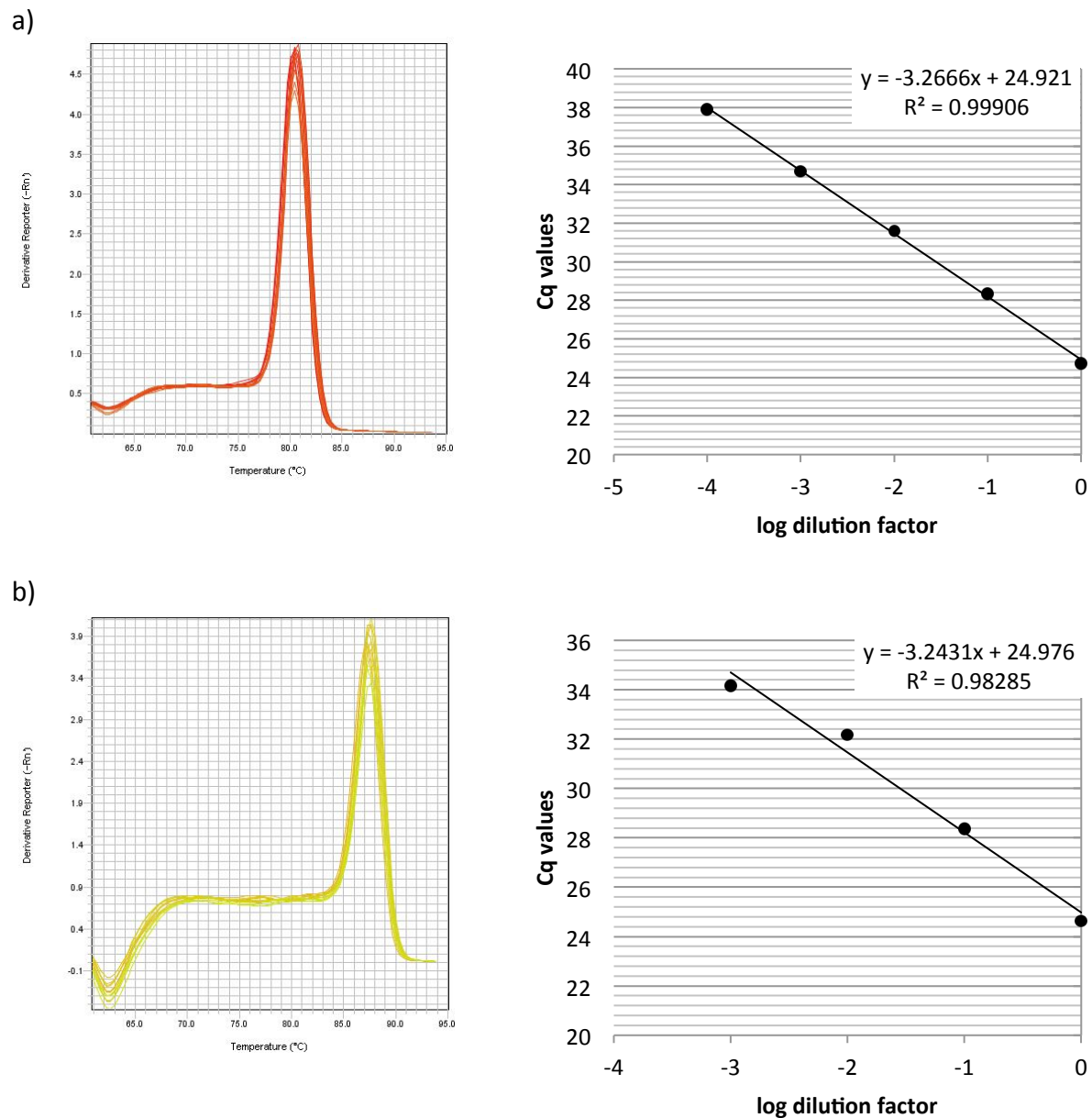


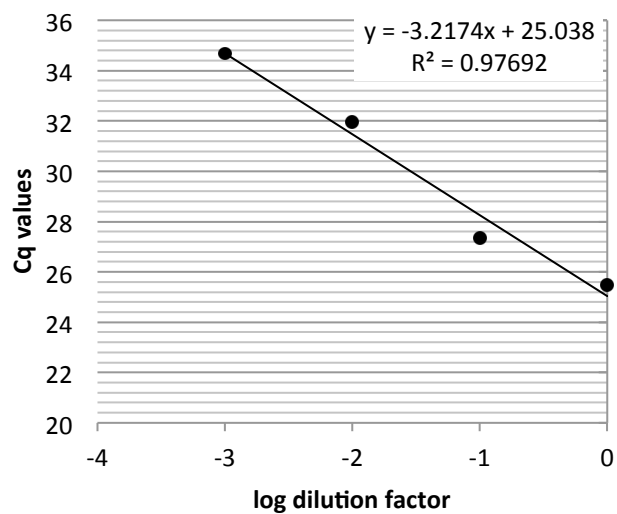
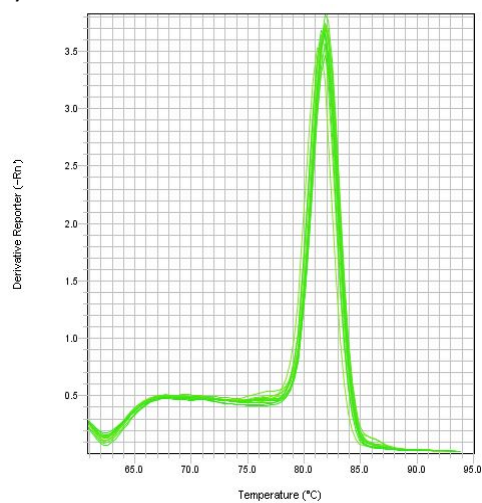
Table 1 Reference genes primer pair sequences and amplicon lengths

Gene	Sequence	Amplicon length (bp)	Accession number
ACTB	F: CCAGACATCAGGGTGTGATGG R: CTCCATATCATCCCAGTTGGTGA	137	AJ719605
B2M	F: TACTCCGACATGTCCTTCAACG R: TCAGAACTCGGGATCCCACTT	150	AB162661
GAPDH	F: GAAGGCTGGGGCTCATCTG R: CAGTTGGTGGTGCACGATG	150	AF047874
GUSB	F: GGCAGACTGGTCCTGTTGTTG R: GGGTCCTGAGTGATGTCATTGA	64	AJ720880
TBP	F: AGCTCTGGGATAGTGCCACAG R: ATAATAACAGCAGCAAAACGCTTG	134	AF221563
TUBAT	F: CAGCTCTCAGTGGCTGAAATCA R: CCTTGTTGCGGGTCACACTT	77	M16030
r28S	F: GGCGAAGCCAGAGGAAACT R: GACGACCGATTGTCACGTC	62	FM165415

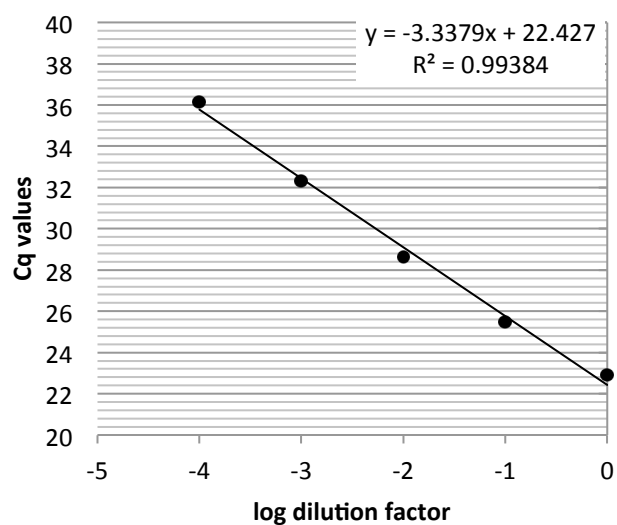
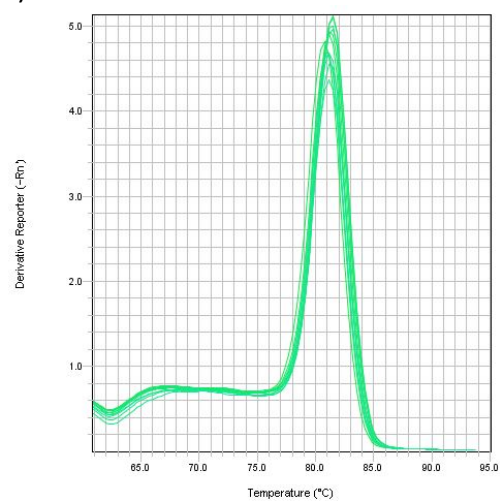
Figure 1. Confirmation of specificity of primer pair products for seven reference genes using melting curve analysis (left panel). For analysis of reaction efficiency, standard curves were generated using log-fold serial dilutions of pooled cDNA from spleen cells, thymus cells and bursa cells (control and stimulated); Data for ACTB (a); B2M (b); GAPDH (c); GUSB (d); TBP (e); TUBAT (f); r28S (g).



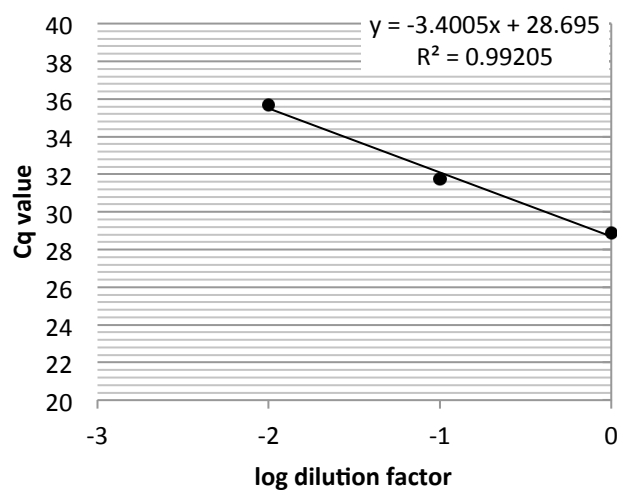
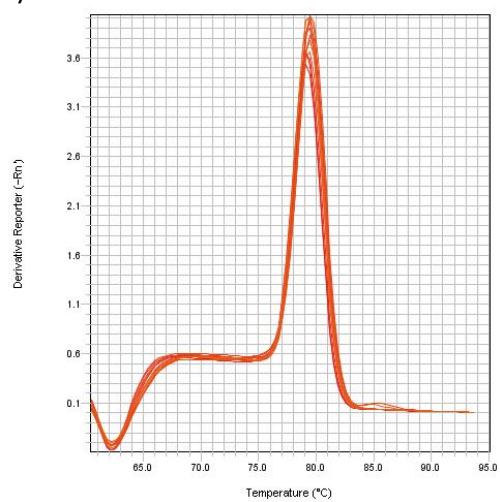
c)



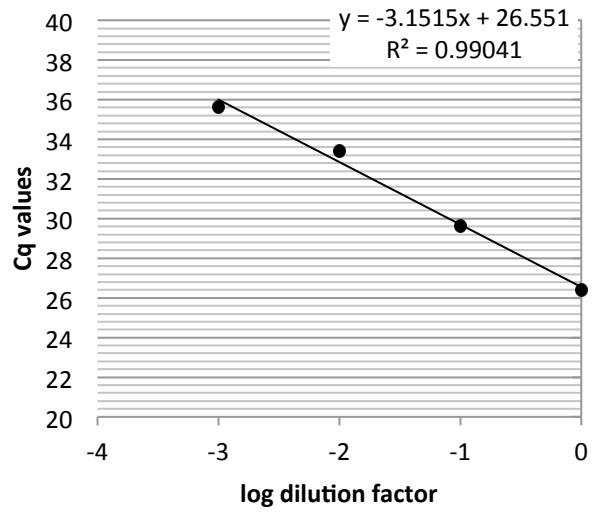
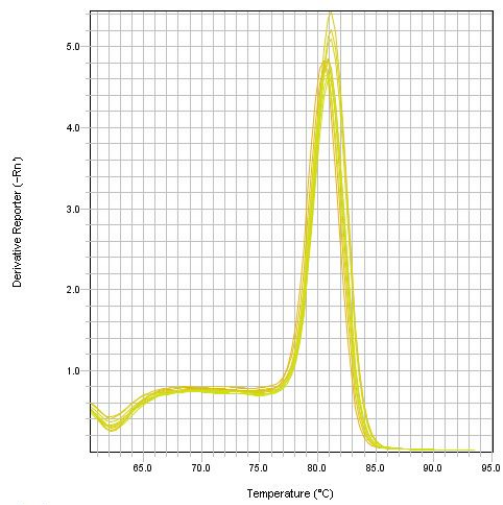
d)



e)



f)



g)

